Site-directed mutagenesis and functional analysis of active site acidic amino acid residues D142, D144 and E146 in *Manduca sexta* (tobacco hornworm) chitinase

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Abstract

Chitinases (EC 3.2.1.14) are glycosyl hydrolases that catalyze the hydrolysis of β-(1, 4)-glycosidic bonds in chitin, the major structural polysaccharide present in the cuticle and gut peritrophic matrix of insects. Two conserved regions have been identified from amino acid sequence comparisons of family 18 glycosyl hydrolases, which includes *Manduca sexta* (tobacco hornworm) chitinase as a member. The second of these regions in *M. sexta* chitinase contains three very highly conserved acidic amino acid residues, D142, D144 and E146, that are probably active site residues. In this study the functional roles of these three residues were investigated using site-directed mutagenesis for their substitutions to other amino acids. Six mutant proteins, D142E, D142N, D144E, D144N, E146D and E146Q, as well as the wild-type enzyme, were produced using a baculovirus-insect cell line expression system. The proteins were purified by anion-exchange chromatography, after which their physical, kinetic and substrate binding properties were determined. Circular dichroism spectra of the mutant proteins were similar to that of the wild-type protein, indicating that the presence of mutations did not change the overall secondary structures. E146 was required for enzymatic activity because mutants E146Q and E146D were devoid of activity. D144E retained most of the enzymatic activity, but D144N lost nearly 90%. There was a shift in the pH optimum from alkaline pH to acidic pH for mutants D142N and D144E with minimal losses of activity relative to the wild-type enzyme. The pH-activity profile for the D142E mutation resembled that of the wild-type enzyme except activity in the neutral and acidic range was lower. All of the mutant proteins bound to chitin. Therefore, none of these acidic residues was essential for substrate binding. The results indicate that E146 probably functions as an acid/base catalyst in the hydrolytic mechanism, as do homologous residues in other glycosyl hydrolases. D144 apparently functions as an electrostatic stabilizer of the positively charged transition state, whereas D142 probably influences the pK_a values of D144 and E146. Published by Elsevier Science Ltd.

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1. Introduction

Chitinolytic enzymes have been isolated from numerous organisms including bacteria, fungi, insects, plants and animals (Koga et al., 1999; Fukamizo, 2000). Based on amino acid sequence alignments and hydrophobic cluster analysis, Henrissat and associates have classified chitinases into families 18 and 19 of glycosyl hydrolases (Henrissat and Bairoach, 1993; Coutinho and Henrissat, 1999). Family 19 chitinases are almost exclusively plant enzymes, and have a high degree of similarity. In contrast, family 18 includes representatives from microbes (bacterial, fungal and viral), plants and animals, and have substantial sequence divergence. In spite of their diverse physical, biological and enzymatic properties and primary sequences, family 18 chitinases have two short, highly conserved sequences, suggesting that these regions might be involved in catalysis and/or substrate binding (Kramer and Muthukrishnan, 1997). One of these regions, conserved region II, is particularly rich in acidic amino acids and has the consensus sequence,

FDGLDLDWEYP. Some of the acidic residues in conserved region II of family 18 hydrolases may be involved in catalysis. These would be analogous to the roles of E35 and D52 in the catalytic mechanism of chicken lysozyme, another glycosyl hydrolase which hydrolyzes chitin oligosaccharides (Sinnott, 1990). This possibility was investigated in a microbial chitinase from *Bacillus circulans* (Watanabe et al., 1993, 1994). These investigators demonstrated that the glutamate residue in this sequence was important for catalysis because replacement of this residue with several other amino acids resulted in loss of enzymatic activity. The aspartic acid residues in this consensus sequence were found to influence the kinetic parameters of this enzyme. A more recent study of a microbial chitinase encoded by the *Autographa californica* nuclear polyhedrosis virus suggested a similar role for the glutamate residue in this conserved sequence, but the roles of the corresponding aspartate residues were not determined (Thomas et al., 2000).

We are investigating how the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae), uses chitinolytic enzymes to digest chitin in the exoskeleton during molting and metamorphosis. The long-term goal of this research is to manipulate molting enzymes for insect pest management (Kramer et al., 1997; Ding et al., 1998). In this paper, we focus on some of the putative active site residues and present the results of a study on the effect of replacing three of the four acidic amino acids, D142, D144 and E146, in the second conserved sequence of *M. sexta* chitinase. Acidic amino acids were replaced with either a different acidic residue or the corresponding amide to investigate effects on pH optima, kinetic parameters and chitin binding of the enzyme. Our data indicate that the glutamate residue is very likely the proton donor in the catalytic mechanism, whereas the two aspartic acid residues influence the optimum pH of the enzyme and, in some cases, the kinetic parameters as well. None of these acidic residues are required for chitin-binding by the enzyme.

2. Materials and methods

2.1. Site-directed mutagenesis

The unique site elimination (U.S.E.) mutagenesis kit (Pharmacia Biotech) was used for site-directed mutagenesis. Three primers based on the sequence for *Manduca* chitinase (Kramer et al., 1993) were utilized in this study. The Genbank accession numbers for *M. sexta* chitinase precursor mRNA is U02270 and S64757. To knock out the two PvuI sites in pBSKS (+) plasmid, two selection primers were designed as follows (Pharmacia protocol): PvuI A: 5′-G T T G G G A A G **G G C A A T T** G G T G C G G G C C T C -3′ and PvuI B: 5′-C T T C G G T C C T C C **A A T T G** T

T G T C A G A A G -3′. The residues in bold represent the sequence that replaced the original PvuI site.

Target primers for generating D142N, D142E, D144N, D144E, E146Q and E146D mutations were: 5′-C TTC GAC GGT CTG **AAC** CTT GAT TGG G-3′, 5′-TTC GAC GGT CTG **GAG** CTT GAT TGG-3′, 5′-GGT CTA GAC CTT **AAT** TGG GAG TAC C-3′, 5′-CTA GAC CTT **GAG** TGG GAG TAC-3′, 5′-C TTT GAT TGG **CAG** TAC CCA GGA GCC-3′ and 5′-C CTT GAT TGG **GAC** TAC CCA GGA GC-3′, respectively. In the primers designed above, codon D142 (GAC) was replaced by AAC (N) or GAG (E), codon D144 (GAT) was changed to AAT (N) or GAG (E), and the GAG codon for E146 was substituted by CAG (Q) or GAC (D). The modified codons are denoted above in bold.

A mixture of 0.025 pmol plasmid template DNA and 1.25 pmol of each phosphorylated primer was used in the DNA synthesis and ligation steps, The reaction mixtures were subjected to PvuI restriction enzyme digestion (10 units) for 1 h. Competent cells from a repair-defective strain of *Escherichia coli* (NM522 *mut*S) were used for transformation and plasmids were isolated and purified from the overnight culture. The plasmid DNA mixture was then subjected to a second round of digestion with PvuI to decrease the proportion of wild-type plasmids with intact PvuI sites. An aliquot of the digested mixture containing about 25 ng plasmid DNA was used to transform 100 µl *E. coli* JM109 competent cells (1×10^{10} cells/ml). After selection in ampicillin-containing medium, amplification and purification, the plasmids resistant to PvuI digestion were isolated and sequenced to confirm that desired site-directed mutagenesis had taken place.

2.2. Expression of mutant forms of chitinase in baculovirus-infected insect cells

From the plasmids that were confirmed by DNA sequencing as having the desired mutations, the 1.8 kb chitinase gene coding fragments were cut out using EcoRI and then inserted at the EcoRI site of the baculovirus transfer plasmid pVL1393 behind the polyhedrin promoter as described by Gopalakrishnan et al. (1995). The recombinant pVL1393-chi DNA was mixed with linearized BaculoGold™ DNA from Pharmingen and co-transfection was carried out according to Huang et al. (2000). The recombinant baculoviruses containing the mutant forms of the *M. sexta* chitinase gene were obtained. The media from co-transfection were used as starting material to obtain high titer virus stocks after amplification in monolayer sf21 cells using serum-containing medium in 25-cm² T-flasks. Protein expression was carried out in monolayers of Hi5 cells using serum-free medium Excell-405 (JRH Biosciences, Lenexa, KS) in 75-cm² T-flasks. The multiplicity of infection was

between 3–5. Infected cells were incubated at 27°C. After 5 h, the medium that contained serum from the virus inoculation stock was removed and 15 ml of fresh serum-free Excell-405 medium was added. After 60 h, the medium was collected and the expression level was determined by SDS-PAGE analysis (9% acrylamide) followed by Coomassie blue staining and western blotting. The medium was stored at −70°C.

2.3. Confirmation of the mutation in the baculoviral DNA by sequencing

Genomic baculovirus DNA was prepared from virus stocks according to O'Reilly et al. (1994). Three ml of high titer virus stock were centrifuged at 12,000 g for 15 min at 4°C to pellet the virus. One hundred μl of virus-disruption buffer (10 mM Tris-HCl, pH 7.6; 10 mM EDTA, 0.25% SDS) was added to the pellet. Proteinase K was added to a final concentration of 500 μg/ml. The mixture was incubated at 37°C for about 2 h until it was clear. DNA was purified by phenol/chloroform extraction and ethanol precipitation. The purified viral genomic DNA was mixed with 5 μM each of the forward primer, 5′-GTC TTT TAT CAG GAG CGT TG-3′, and the reverse primer, 5′-AGA ACT AGT TAC CCA AGA AG-3′, to carry out PCR amplification of *M. sexta* chitinase gene-coding fragments that included the region of mutagenesis. The DNA sequence of the PCR-amplified product (800 bp) was determined by Integrated DNA Technologies, Inc. (Coralville, IA) using the same forward primer.

2.4. Purification of chitinase

After centrifugation (1,000 g, 10 min) to remove cell debris, the culture medium (50 ml) was dialyzed against 5 L of 20 mM sodium phosphate buffer (pH 7.0) overnight using a 30 kDa cutoff membrane tubing. A DEAE-Sepharose (Sigma) column (1.5×20 cm) was equilibrated with the same buffer (pH 7.0). After the protein was loaded, the column was washed with the same buffer and then eluted with a NaCl gradient from 0 to 0.35 M in a total volume of 140 ml at a rate of 2 ml/min. The absorbance of every other 2-ml fraction was measured at 280 nm. The fractions were also analyzed by SDS-PAGE. The fractions containing the 85 kDa proteins (expected for *M. sexta* chitinase) were pooled and concentrated using a Centriprep® filter (Amicon) to a final concentration of approximately 1 μg/μl.

2.5. Enzyme assays and determination of kinetic parameters

Chitinase activity as a function of pH was determined using both carboxymethyl-chitin-Remazol brilliant violet (CM-Chitin-RBV) and 4-methylumbelliferyl-β-D-N, N′,

N″-triacetylchitotriose [MU-(GlcNAc)$_3$] as substrates. For the polymeric substrate, 0.5 μg protein was diluted to 200 μl with water and incubated with 100 μl of CM-Chitin-RBV (2 mg/ml) and 100 μl of 0.2 M universal buffer, pH 3.1–11.7, at 37°C for 2 h. To stop the reaction, 100 μl of 2 M HCl was added. For the oligosaccharide substrate, 33.5 μl protein (0.1 μg) was mixed with 4 μl MU-(GlcNAc)$_3$ (1.5 mM) and 12.5 μl universal buffer (0.2 M), pH 3.1–11.7. The reaction was incubated at 37°C for 15 min. To stop the reaction, 12.5 μl of 2 M HCl was added.

Chitinase activity as a function of temperature was determined using CM-Chitin-RBV as the substrate and following a procedure similar to that for determining the activity-pH profile except for using temperature as the variable. The temperature ranged from 17 to 77°C.

Kinetic parameters were obtained using both a polymeric substrate, carboxymethyl Remazol Brilliant Violet-chitin (CM-Chitin-RBV, Loewe Biochemica, Munich, Germany) and an oligosaccharide substrate, 4-methylumbelliferyl β-N, N′, N″-triacetylchitotrioside [MU-(GlcNAc)$_3$, Sigma], as described by Huang et al. (2000). In the CM-Chitin-RBV assay, 200 μl of enzyme (0.5 μg), 100 μl of buffer (at the optimal pH for each enzyme) and 100 μl of CM-Chitin-RBV (ranging from 0.02 to 0.3 mg/ml) were mixed together and the reaction was carried out at 37°C for 15 min. The enzyme activity unit was ΔA_{520}/μg/h.

In the MU-(GlcNAc)$_3$ assay, the reaction was carried out at 37°C at pH 6.3 (Frugoni, 1957) for 10 min. The reaction was conducted by mixing 12.5 μl of 0.2 M universal buffer, containing acetate, borate and phosphate, with 0.1 μg protein and MU-(GlcNAc)$_3$ (ranging from 6 to 300 μM) to a final volume of 50 μl. After the reaction, the products were mixed with 1.94 ml 0.15 M glycine-NaOH buffer (pH 10.5). The released fluorescent methylumbelliferone (MU) was measured by fluorescence spectrophotometry (DyNA Quant 200 Fluorometer, Hoefer) (Hollis et al., 1997). The fluorescence intensity was proportional to the released products. A standard curve was used to determine the quantity of the products. The enzyme activity unit was nmol product/μg protein/min.

The kinetic parameters, K_m and k_{cat}, were calculated using the nonlinear least-square fitting procedure for the Michaelis–Menten Eq. in Prism software (GraphPad Prism version 3.0 for Windows, San Diego, CA).

2.6. Chitin binding assay

A modified binding assay protocol was used to study the binding ability of the purified wild-type and mutant forms of *M. sexta* chitinase toward the insoluble substrate. Chitin was prepared according to Kuranda and Robbins (1991) and Venegas et al. (1996). Fifty mg of ground chitin (Sigma) was boiled for 5 min in a 1 ml

solution containing 1% SDS and 1% β-mercaptoethanol (β-ME) and then washed with 15 ml of water. After several washes and filtering (0.45 μm filter), the chitin was resuspended in 1 ml of 20 mM phosphate buffer (pH 6.5) containing 0.2% NaN₃. One hundred μl of the above suspension was centrifuged and washed twice with the same buffer without NaN₃. Two μg of the purified chitinase protein (150 μl) were mixed with the chitin pellet. The mixture was incubated at room temperature for 1 h with constant shaking. Then, it was centrifuged at 12,000 g for 15 min and the supernatant was collected as the unbound fraction. The pellet was washed twice with 75 μl of 20 mM sodium phosphate buffer (pH 6.5) and the wash fractions were pooled. After the wash, the pellet was resuspended in 120 μl of 20 mM sodium phosphate buffer and 30 μl of 5×protein SDS gel loading buffer was added to suspension. The mixture was boiled for 5 min and then centrifuged at 12,000 g for 10 min. The supernatant containing the bound fraction was collected. Fifty μl of each fraction was subjected to SDS-PAGE.

2.7. Circular dichroism (CD)

Proteins were diluted to 0.58 μM (0.049 mg/ml) in 410 μl of 20 mM sodium phosphate buffer (pH 6.8). CD spectra over 5 scans were measured using a Jasco J-720 spectropolarimeter at 20°C (Greenfield, 1996; Sreerama and Woody, 2000). After noise reduction and concentration adjustment, the ellipticity was converted to the molar ellipticity, which was plotted vs wavelength.

2.8. Allosamidin inhibition assay

Following the same procedure as outlined above in the section on activity-pH profile for CM-Chitin-RBV as the substrate, allosamidin was added to the reaction mixture at concentrations ranging from 0.005 to 30 mM at the optimal pH of each protein. The control was the reaction without inhibitor (allosamidin). The relative enzyme activity versus the control was the measure of the extent of inhibition.

3. Results

3.1. Protein purification

Six mutations in the *M. sexta* chitinase cDNA clone 10 (Kramer et al., 1993) involving the active site aspartate residues (D142 and D144) and the glutamate (E146) were generated and used to obtain the recombinant baculoviruses as described in Materials and Methods section. The baculoviruses with the *M. sexta* chitinase genes carrying the D142E, D142N, D144E, D144N, E146D and E146Q mutations were used to obtain several mgs each of the corresponding mutant. The proteins were purified

from the culture medium by DEAE-cellulose anion-exchange chromatography and were >95% pure as revealed by SDS-PAGE followed by staining with Coomassie blue (data not shown). The purified proteins were used for study of their enzymatic and structural properties.

3.2. Changes in acidic amino acids alter the shapes of the pH-activity profiles

The pH vs activity profiles of the six mutant enzymes were determined over the range of pH 3–11.7 using either a polymeric or oligosaccharide substrate. With both substrates, the wild-type enzyme had the broadest pH range of activity when compared with the mutant forms. The shapes of the pH-activity profiles of the mutant enzymes were altered, the results of which are described in detail below.

3.2.1. Polymeric substrate and D142E and D142N

The D142E mutant protein, which retained the side-chain carboxyl group and also contained an extra methylene group, had nearly the same activity as the wild-type enzyme in the alkaline range (>pH 8.5), but it had reduced activity at neutral and acidic pH values (Fig. 1A). The overall range of pH over which the enzyme was active, however, was not substantially affected. The pH optimum was nearly the same as the wild-type enzyme (pH 8.5–9.0). Thus, this mutation had only a minor effect on the activity of the enzyme. On the other hand, the D142N mutation, which lacked a side chain carboxylate group, had a dramatically narrowed pH range of activity and an acidic instead of an alkaline pH optimum (pH 4.5). The right side of the pH-activity curve was shifted by about 2 pH units to the acidic side. The mutant enzyme essentially had no activity above pH 8.5. Nonetheless, the activity at pH 5.4 was about the same as that of the wild-type enzyme.

3.2.2. Polymeric substrate and D144E and D144N

With the D144E mutation, enzyme activity was very low above pH 8.5, unlike the wild-type or D142E enzymes (Fig. 1A). Its pH optimum was shifted to the acidic range, similar to the D142N enzyme. Its activity, however, was only about 30% of the wild-type enzyme (compare V_{max} values in Table 1). The D144N mutation exhibited an even more drastic effect on enzyme activity. It had low activity, only about 10% of the wild-type enzyme, with no clear-cut pH optimum. Thus, mutations of D144 were generally more detrimental to *M. sexta* chitinase than those involving D142.

3.2.3. Polymeric substrate and E146D and E146Q

Both of these mutant enzymes had no detectable activity over the entire range of pH values tested (Fig. 1A). Thus, E146 was essential for activity and cannot
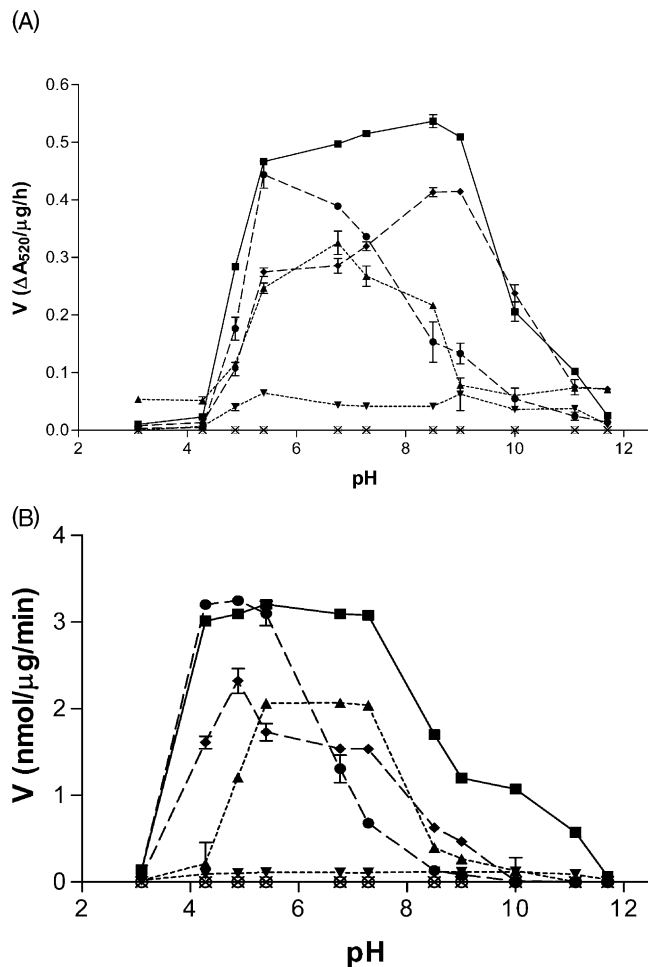
(A)

(B)

Fig. 1. (A) Activity vs pH profiles of wild-type and mutant forms of *M. sexta* chitinase using the polymeric substrate, CM-Chitin-RBV. The activities of wild-type and mutant chitinases (1.25 μg/ml) were measured with 0.5 mg/ml substrate in 0.2 M universal buffer at the indicated pH values (total volume 0.4 ml) at 37°C for 2 h. Less than 10% of the substrate was digested even though a relatively long incubation time was utilized. Wild-type (■), E146Q (○), E146D (X), D144E (▲), D144N (▼), D142E (◆), and D142N (●). Standard deviations (S. D.) are shown for each sample as vertical bars above and below the mean values (*n*=3). In many cases, the vertical bars are not visible due to a very small S. D. (B) Activity vs pH profiles of wild-type and mutant forms of *M. sexta* chitinase using the oligosaccharide substrate, MU-(GlcNAc)$_3$. The activities of wild-type and mutant chitinases (0.002 mg/ml) were measured with 120 μM substrate in 0.2 M universal buffer (total volume 0.05 ml) at 37°C for 15 min. Wild-type (■), E146Q (○), E146D (X), D144E (▲), D144N (▼), D142E (◆), and D142N (●). Standard deviations (S. D.) are shown for each sample as vertical bars above and below the mean values (*n*=3). In many cases, the vertical bars are not visible due to a very small S. D.

be substituted for even by an aspartate with a side chain that lacks only one methylene group relative to the wild-type enzyme's glutamate side chain.

3.2.4. Oligosaccharide substrate

To determine whether the activities of the mutant enzymes differed when acting upon an oligosaccharide substrate, which may bind to fewer sub-sites in the bind-

ing cleft of the enzyme than the polymeric substrate, the pH versus activity profiles were determined using the modified trisaccharide, MU-(GlcNAc)$_3$ (Fig. 1B). As was observed with the polysaccharide substrate, the wild-type chitinase exhibited the broadest pH range of activity (pH 3.5–11) with the chitin oligosaccharide. All of the mutant enzymes had a narrower pH range with very low or undetectable activity in the alkaline range (above pH 9.0). The effects of the individual mutations on the activity of the enzymes with the oligosaccharide substrate were similar to those seen with the polymeric substrate with the exception of the D142E enzyme, which also exhibited a shift in the pH optimum to the acidic side. As was seen with the polymeric substrate, the activity of D142E mutation was affected the least. The D144N, E146D and E146Q mutations exhibited little or no activity when using the fluorogenic trisaccharide as the substrate.

3.3. Temperature profile

Activities of the wild-type and mutant enzymes as a function of temperature were determined as described in the Materials and Methods section. The wild-type and D142E enzymes had the highest activity at 37°C, whereas D142N and D144E enzymes had an optimal temperature of about 45°C (data not shown). The D144N enzyme was most active at 57°C. All of the enzymes had little or no activity above 67°C.

3.4. Kinetic parameters of mutant enzymes

Both the oligosaccharide, MU-(GlcNAc)$_3$, and the polysaccharide, CM-Chitin-RBV, were used as substrates to measure the kinetic parameters, K_m and k_{cat}, for the mutant enzymes at the optimum pH of each enzyme. Table 1 shows that both the K_m and k_{cat} values of each mutant enzyme towards the oligosaccharide substrate were different from those of the wild-type enzyme. Most of mutants had lower k_{cat} values except for the D142N enzyme. The k_{cat} values of D144E and D144N were affected the most. D144N had the lowest k_{cat} value. With the polysaccharide substrate, the k_{cat} value was reduced 8-fold, whereas with the MU-(GlcNAc)$_3$ substrate, the reduction was nearly 250-fold. Interestingly, the D144E and D144N mutations appeared to increase the affinity of the enzyme for the substrate as the K_m values were substantially lower than that of the wild-type enzyme. However, the K_m for the D142N enzyme was slightly increased. The K_m values for the oligosaccharide followed the same pattern as observed with the polysaccharide with the exception of the D144N enzyme having a >200-fold lower K_m than the wild-type enzyme. However, the k_{cat}/K_m ratios for all of the mutant forms changed relatively little because of compensating

Table 1
Kinetic parameters of *M. sexta* chitinases using CM-Chitin-RBV (A) and MU-(GlcNAc)$_3$ (B) as substrates

(A)

Parameter	Enzyme				
	Wild-type	D142E	D142N	D144E	D144N
$k_{cat} \times 10^6$ (ΔA_{520}/mol/s)	64.5	46.1	76.3	20.2	8.0
K_m (mg/ml)	0.17	0.15	0.23	0.04	0.03
$k_{cat}/K_m \times 10^6$ (ΔA_{520}/mol/s/mg/ml)	379.5	307.8	331.8	493.4	266.6

(B)

	Wild-type	D142E	D142N	D144E	D144N
k_{cat} (s^{-1})$\times 10^{-2}$	370.0	188.0	650.0	165.0	1.5
K_m (μM)	234.5	102.3	506.7	118.2	1.0
$k_{cat}/K_m \times 10^4$ (M^{-1} s^{-1})	1.6	1.8	1.3	1.4	1.5

decreases in K_m values when k_{cat} values were lower relative to the wild-type enzyme.

3.5. Inhibition by allosamidin

Allosamidin, a pseudotrisaccharide isolated from *Streptomyces* sp. (Sakuda et al., 1987), contains two N-acetylglucosamines attached to allosamizoline. It is a potent inhibitor of several insect and microbial chitinases and the plant chitinolytic enzyme, hevamine, another family 18 chitinolytic enzyme (Koga and Isogai, 1987; Koga et al., 1999). The structural similarity of allosamidin with the proposed transition state oxazoline intermediate of family 18 glycosidases makes this compound a specific inhibitor of this family. Depending on the protein, the following inhibitor concentration ranges were used for a concentration-response analysis: 0.005–0.2 μM for wild-type and D142E; 0.1–15 μM for D144E; and 0.1–28 μM for D144N. The enzymatic activities of mutant chitinases at different allosamidin concentrations were measured relative to the wild-type enzyme, as described in the Materials and Methods section. The IC$_{50}$ values (the concentration of inhibitor at which the activity is half that of the reaction without inhibitor) was used to compare the inhibition efficiency for each enzyme. Table 2 shows the IC$_{50}$ values for the wild-type and mutant enzymes. These results demonstrate that the wild-type enzyme bound most tightly with the inhibitor.

Table 2
Allosamidin inhibition (IC$_{50}$, μM) of wild-type and mutant *M. sexta* chitinases

Enzyme	pH 5.40	pH 6.76	pH 8.40
Wild-type	0.05	0.02	0.01
D144E	–	8.09	–
D144N	–	16.34	–
D142E	–	–	0.01
D142N	0.50	–	–

The IC$_{50}$ value of D142N was nearly ten times greater than that of the wild-type enzyme, but the most dramatic change was observed with the D144N and D144E mutants. The IC$_{50}$ value of D144N was almost 800 times greater than that of wild-type, and that of D144E was 400-fold greater, indicating that D144 greatly influences the binding ability of the enzymes to the transition state. Modification of D142, on the other hand, had a lesser effect on the IC$_{50}$ for allosamidin. Obviously, loss of the carboxylate group of D144 or alteration of its location had a greater impact on binding than any changes in other residues.

3.6. Chitin binding

The binding ability of the purified wild-type and mutant forms of *M. sexta* chitinase toward the insoluble substrate, chitin, was determined as described in the Materials and Methods section. The results showed that neither the wash nor supernatant fractions contained protein, indicating that binding to chitin had occurred with all of the proteins. Only by harsh treatment with an SDS solution could any of the proteins be eluted from the chitin pellet (Fig. 2). This result indicated that none of the mutant proteins studied had a detectable change in chitin binding.

3.7. Circular dichroism spectra

The possibility that the overall secondary structure of the protein might have changed because of a mutation was checked by circular dichroism (CD) analysis. As shown in Fig. 3A, the wavelength scan between 240 and 195 nm resulted in spectra with absorbance minima at approximately 220 and 208 nm. The CD spectra at pH 6.5 of D142E, D142N, D144E, D144N, E146D and E146Q were very similar to that of the wild-type enzyme, suggesting that the presence of mutations did not change the overall structures at pH 6.5. These spectra exhibit features characteristic of a protein that contains
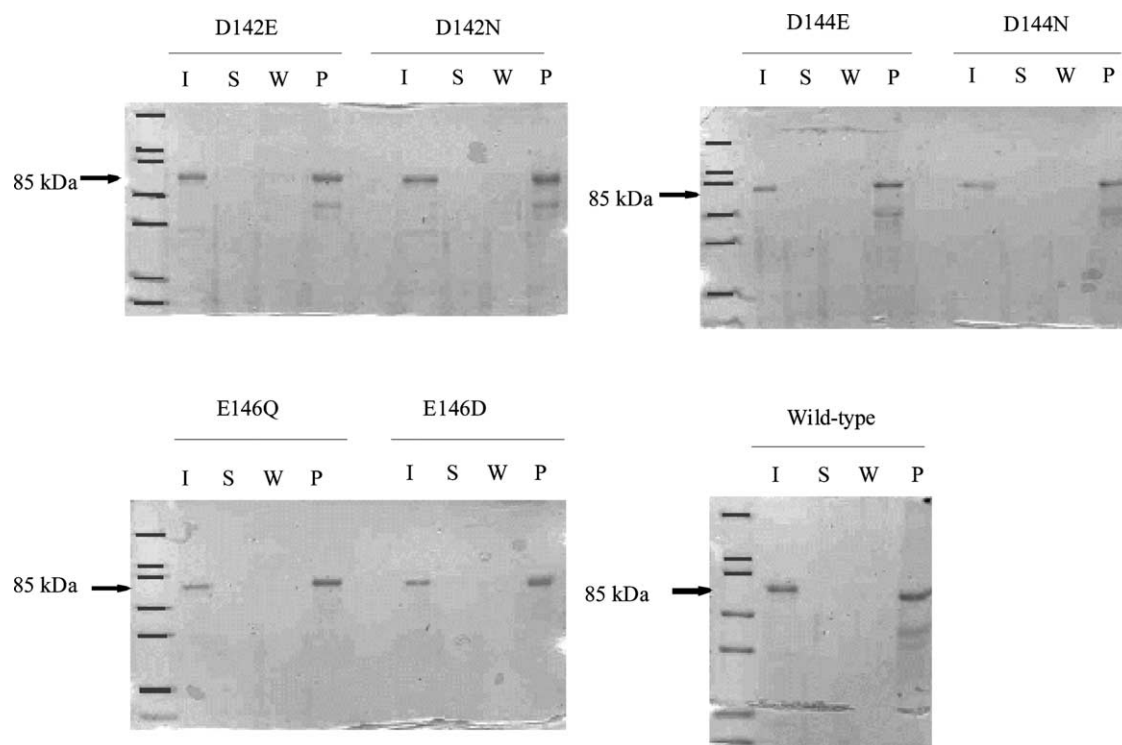
Fig. 2. SDS-PAGE of proteins from chitin binding assay for wild-type and mutant forms of *M. sexta* chitinase. The assay was carried out as described in the Materials and Methods section. Protein (2 μg) was incubated with chitin (50 mg in 150 μl) at room temperature for 1 h. I, initial fraction; S, supernatant; W, wash fraction; and P, SDS-eluant from pellet fraction. A control assay to check for nonspecific binding using 2 μg of bovine serum albumin resulted in the complete recovery of this protein in the wash (W) fraction (data not shown).

both α–helices and β–sheets, which was expected for this $(\beta\alpha)_8$-barrel folded protein (Huang et al., 2000; Bondesen and Schuh, 2001). Because some of the mutant enzymes showed a loss of activity at around pH 9, CD spectra at pH 9.1 also were obtained to check whether structural differences between wild-type and mutant forms of the enzyme had occurred at that pH (Fig. 3B). The results showed that all of the CD spectra were similar, indicating that the overall structures of wild-type and mutants were very similar at alkaline pH as well.

4. Discussion

4.1. M. sexta *chitinase utilizes an oxazoline intermediate in the transition state*

Nature has produced an impressive assortment of hydrolytic enzymes belonging to nearly 85 families of glycosidases to cope with the large number of chemically and structurally different glycosides (for more information, visit the Carbohydrate Active Enzymes web site server at http://afmb.cnrs-mrs.fr/~pedro/CAZY/). Since the time when the crystal structure of lysozyme was resolved (Phillips, 1966), the catalytic mechanisms of glycosidic enzymes have been studied intensively.

The catalytic mechanism of chitinase was first suggested to be similar to that of hen's egg white lysozyme, which utilizes a carbonium ion intermediate . With more structural data available regarding different chitinases, however, other hypotheses concerning catalytic mechanisms were proposed including a single-displacement "retaining" reaction with an oxocarbenium ion transition state (TS), a "retaining" double-displacement reaction with an oxazoline TS, or an "inverting" double-displacement reaction involving both an oxocarbenium ion and a bound water in the TS . *Bombyx mori* chitinase, which is closely related to *M. sexta* chitinase in sequence and other properties, was found to utilize a "retaining" mechanism, producing products that retain the β-anomeric configuration (Fukamizo et al., 1995; Banat et al., 1999). All of the enzymes of this family examined so far are inhibited by allosamidin, an aglycone allosamizoline analog of oxazoline, which is the transition state intermediate proposed for family 18 chitinases (Brameld et al., 1998). Additional evidence supporting such a mechanism was obtained from the crystal structures of two group 18 chitinases and four other related proteins (summarized in Van Scheltinga et al., 1996). The crystal structure of a complex between allosamidin and hevamine, also a family 18 chitinase, revealed that loops at the carboxyl ends of the two beta-sheets containing the two conserved regions form part of the substrate binding cleft
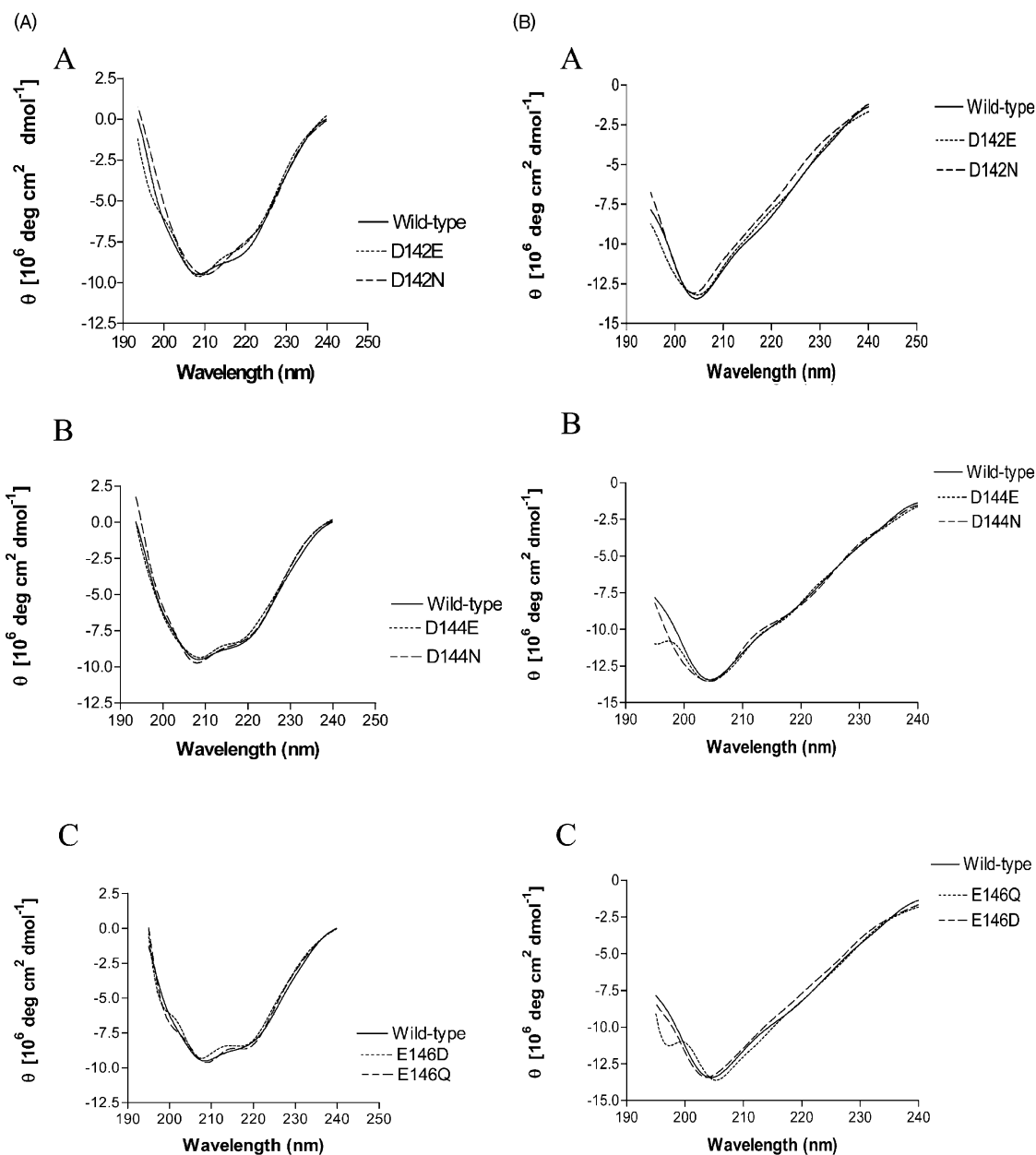
Fig. 3. (A) Circular dichroism spectra of wild-type and mutant forms of *M. sexta* chitinase. CD spectra were measured in 20 mM phosphate buffer, pH 6.5 at room temperature in a 0.1 cm cuvette. (A) Wild-type, D142E, and D142N enzymes. (B) Wild-type, D144E, and D144N enzymes. (C) Wild-type, E146D, and E146Q enzymes. Protein concentration is 0.58 μM. (B) Circular dichroism spectra of wild-type and mutant forms of *M. sexta* chitinase. CD spectra were measured in 20 mM phosphate buffer, pH 9.1 at room temperature in a 0.1 cm cuvette. (A) Wild-type, D142E, and D142N enzymes. (B) Wild-type, D144E, and D144N enzymes. (C) Wild-type, E146D, and E146Q enzymes. Protein concentration was 0.58 μM.

(Van Scheltinga et al., 1996). In fact, the conserved glutamate is very near to sub-sites −1 and +1 in the binding cleft (Van Scheltinga et al., 1995). *M. sexta* chitinase contains the two conserved signature sequences of family 18 chitinases and is inhibited by allosamidin, suggesting that it very probably utilizes a "retaining" double-displacement reaction with an oxazoline TS. Results obtained from homology modeling of *M. sexta* chitinase also are consistent with such a reaction mechanism (Kramer and Muthukrishnan, 1997; Huang et al., 2000). Recently, a new modified "substrate assisted" catalysis

mechanism for chitinolytic enzymes such as insect chitinase was proposed where a water molecule that is hydrogen bonded to the NH of the acetamido group also participates in the hydrolysis (Papanikolau et al., 2001).

4.2. Site-directed mutagenesis of family 18 chitinases

Previously, the roles of glutamic and aspartic acids in conserved region II of five other family 18 chitinases were investigated (Table 3), including the bacterial chitinases from *S. marsecens* (Papanikolau et al., 2001), *B.*

Table 3
Comparison of the enzymatic activities of different family 18 chitinases of DxDxE motif

| | D | | | D | | | E | | | |
	D→G	D→E	D→N	D→A	D→E	D→N	E→D	E→Q	E→G	Reference
Chitinase (*Manduca sexta*)	–	Moderate activity D142E	Moderate D142N	–	Moderate D144E	Low D144N	Inactive E146D	Inactive E146Q	–	This work
Chitinase A1 (*Bacillus circulans*)	–	Same activity as wild-type D200E	Low D200N	–	Low D202E	Low D202N	Inactive E204D	Inactive E204Q	–	Watanabe *et al.*, 1993, 1994
Chitinase (*Alteromonas sp.*)	–	–	Moderate activity D288N	–	Moderate D290E	Inactive D290N	Inactive E292D	Inactive E292Q	–	Tsujibo *et al.*, 1996
Chitinase (*Aeromonas caviae*)	–	–	–	–	Low activity D313E	Moderate D313N	Inactive E315D	Inactive E315Q	–	Lin et al., 1999
Chitinase[a] (*Autographa californica*)	Increased activity[b] D311G	–	–	–	–	–	–	–	Moderate E315G	Thomas et al., 2000
Chitinase A (*Serratia marcescens*)	–	–	–	Inactive D313A	–	–	–	Inactive E315Q	–	Papanikolau et al., 2001

[a] Crude enzyme preparation was used.
[b] Increased endochitinase activity but a moderate decrease in exochitinase activity was observed.

Species:	Residue Nos.	Sequence
M. sexta	138–148	FDGLDLDWEYP
B. circulans	196–206	FDGVDLDWEYP
A. sp.	284–294	YDGVDIDWEFP
A. caviae	307–317	FDGVDI DWEFP
A. californica	307–317	FDGVDI DWEFP
S. marcescens	307–317	FDGVDIDWEFP
		DxDxE

circulans WL-12 and *Alteromonas* sp. (Tsujibo et al., 1996); *A. californica* nuclear polyhedrosis viral (AcMNPV) chitinase; and the fungal chitinase from *Aeromonas caviae*. The former two chitinases also have been studied by site-directed mutagenesis and characterization of the kinetic parameters of the purified enzymes (Watanabe et al., 1993, 1994; Papanikolau et al., 2001). These studies have confirmed that the glutamate in the conserved motif FDGL**DLD**W**E**YP is most critical for catalysis and that the aspartate residues play less critical but nonetheless important roles. In this study, we have explored in detail the functional roles of the glutamate and the two aspartic acid residues closest to this glutamate in the linear amino acid sequence of *M. sexta* chitinase by a series of site-directed mutations. The use of the baculovirus-insect cell line expression system has permitted us to isolate rather large amounts of each of the mutant proteins free of contamination from wild-type or viral chitinases. The endogenous AcMNPV chitinase has a mass of 60 kDa and is not secreted into the medium (unpublished data; Thomas et al., 2000). The mutant proteins have a mass of approximately 85 kDa and are secreted from the cells.

4.3. E146 in M. sexta *chitinase is the acid/base catalyst*

Results obtained using the E146Q and E146D mutations provided important information about the role of E146 in catalysis and about the possible mechanism used by *M. sexta* chitinase. Replacement of E146 by a sterically similar amide Q led to inactivation. This finding indicated that the carboxyl group of E146 is essential for catalysis. The correct geometry of the carboxyl group of E146 with respect to the cleaved glycosidic bond in the bound substrate also is critical for the catalytic ability of this enzyme, because E146D is also devoid of activity. Similar results were observed with chitinases from *B. circulans*, *Alteromonas* sp. (Tsujibo et al., 1996), AcMNPV, and *A. caviae* when the conserved E in the D×D×E motif (Table 3) was changed. E146 is very likely the proton donor acting as a general acid in *M. sexta* chitinase catalysis. E146 probably participates in the first and most crucial step in the catalytic mechanism, the protonation of the susceptible glycosidic bond, and also in the second step as a proton acceptor from water. The location of the glutamate side chain carboxyl group relative to the glycosidic bond that is cleaved, as revealed by crystal structures of two other members of family 18 chitinases from *S. marcescens* and rubber latex (Perrakis et al., 1994; van Scheltinga et al. (1996), also is consistent with the role of E146 as the acid/base catalyst in *M. sexta* chitinase. The two E146 mutations essentially converted the enzyme into a carbohydrate binding protein devoid of enzymatic activity, which was observed in several naturally-occurring non-enzymatic

chitinase-like proteins such as lectins and inflamation-associated proteins (Huang et al., 2000). The novel mammalian lectin Ym1, which shares significant homology with family 18 glycosyl hydrolases but is enzymatically inactive, is similar to our E146Q protein in that the corresponding residue in Ym1 is Q140 (Sun et al., 2001). In fact, *M. sexta* also produces in hemolymph a nonenzymatic protein homologous with chitinase, which inhibits hemocyte aggregation (Kanost et al., 1994). In that protein A and Q are the residues present in the amino acid sequence which correspond to D144 and E146.

4.4. Role of D144 in catalysis

The D144E mutation caused only a partial loss of enzymatic activity (70%), suggesting that the spatial position of the carboxyl group of D144 is not as critical as that of E146. The carboxylate side chain of the glutamate residue at position 144 of this mutant may function as an electrostatic stabilizer of the transition state in a manner similar to the aspartic acid residue (D144) of the wild-type enzyme. Even the D144N chitinase did not lose all of the enzymatic activity, indicating that this residue is not essential for catalysis. It is likely that D144 helps to stabilize the oxazoline ion intermediate that is formed when the carbonyl oxygen of the N-acetyl group reacts with the C-1 of N-acetylglucosamine at the −1 position of the substrate during the reaction. This role would be similar to that proposed for comparable aspartate residues in two other glycosidic enzymes, a β-hexosaminidase (Hoa and Stephen, 1999) and a chitinase (Van Scheltinga et al. (1995); Brameld et al. (1998); Mark et al. (2001), in the charge stabilization of their transition states. This mechanism also is supported by the observation that *M. sexta* chitinase and the closely related *B. mori* chitinase are inhibited by allosamidin, an oxazoline analog. The partial loss of activity of the D144E and D144N mutants suggests that even though the reaction may proceed via substrate-assisted stabilization of the oxazoline intermediate, D144 apparently also facilitates the reaction by participating in an electrostatic interaction, which further stabilizes the transition state. The progressively lower activities of D144E (30%) and D144N (10%) chitinases relative to the wild-type enzyme are consistent with this hypothesis (compare k_{cat} values listed in Table 1).

Similar results were obtained with *Alteromonas* sp. strain O-7 chitinase with the D290E mutation (equivalent to D144E mutation in *M. sexta* chitinase). There was a loss of 80% of the activity with this mutation, whereas with the D290N mutation, the loss was >99% (Tsujibo et al., 1996). These results are slightly different from those observed with *B. circulans* WL-12 chitinase. In that study, the bacterial enzyme only had very low activity (about 5%) when the residue

homologous with D144 in *M. sexta* chitinase was mutated to E or N. On the other hand, the D313E mutant of *A. caviae* chitinase (corresponding to D144E in *M. sexta* chitinase) lost nearly all activity, whereas the D313N enzyme still had 50–90% activity depending on the substrate hydrolyzed (Table 3). In all of these enzymes, the aspartic acid residue closest to the glutamic acid proton donor has been proposed to act as an electrostatic stabilizer of the oxazoline intermediate . Therefore, the chemical basis of the subtle differences among equivalent mutations of different chitinases is not obvious.

Besides affecting the overall activity, the D144E mutation resulted in a shift by about 2 pH units in the optimum pH value of the *M. sexta* enzyme to the acidic range. In our chitinase model (Huang et al., 2000), the D144E mutation results in the shift of the side chain carboxyl group of D144E from a position close enough to hydrogen bond to D142 to a position where it can form a hydrogen bond with E146. The same change also was observed in the structure of *S. marcescens* chitinase A (used as the template in our chitinase model building), in which the proton donor E315 is located in a hydrophobic pocket lined with residues F191, F316, M388 and W275 (Perrakis et al., 1994). This shift in hydrogen bonding probably disrupts the hydrophobic environment of E146 in *M. sexta* chitinase and lowers its abnormally high pK_a to the acidic side. This hypothesis is consistent with our previous observations that replacement of W145, which is located between D144 and E146, with other hydrophobic amino acids such as F, H, and L also results in a shift in its pH optimum to the acidic side (Huang et al., 2000; unpublished data).

4.5. Role of D142 in catalysis

The D142E mutant of *M. sexta* chitinase had nearly the same activity as the wild-type enzyme, which is similar to the result obtained from the *B. circulans* WL-12 chitinase mutation study involving residue D200. D200E retained 50–80% of the activity of the wild-type enzyme depending on the substrate utilized. However, the D200N mutant of *B. circulans* chitinase had low activity (4–15% of the wild-type enzyme; Watanabe et al., 1993), whereas the comparable mutation (D142N) in *M. sexta* chitinase did not reduce activity quite as much (Table 3). In fact, there was a measurable increase in activity of D142N with the oligosaccharide substrate (Fig. 1B). *Alteromonas* chitinase with the equivalent D288N mutation had about 40% of the activity of the wild-type enzyme (Tsujibo et al., 1996). These data indicate that there are subtle differences in the geometry or in the interactions of charged residues and/or hydrogen bonding among the residues in the catalytic site of the different chitinases.

The hydrogen bonding between D142 and D144 in *M. sexta* chitinase may be important for maintaining the optimal pH of this enzyme as these two residues are lined on the same side of sheet β_4 along with E146, the presumptive acid/base catalyst (Huang et al., 2000). A similar hydrogen bonding triad of carboxylate groups is present in other glycosidase families such as xylanases and α-amylases (Joshi et al., 2000). The β-1,4-glucanase from *Cellulomonas fimi* exhibits this kind of hydrogen bonding network, which is not only responsible for maintaining the proper ionization states of active site residues but also for keeping their correct orientation relative to the cleavage site . If, indeed, there is hydrogen bonding between D142 and D144, mutations of D142 in *M. sexta* chitinase may influence the ionization state of D144. The change in pK_a of D144 will then have an effect on the pK_a of its neighboring group E146. Such a pK_a shift would be expected to change the optimal pH of the enzyme. Indeed, it was observed that the optimal pH shifted to the acidic range when the D142N enzyme was assayed using the polysaccharide substrate. With the oligosaccharide substrate, there was a shift in pH optimum to the acidic range for both the D142N and D142E enzymes.

It happens then that the role of D142 in the *M. sexta* enzyme is to help maintain the required pK_a values of D144 and E146. However, the optimum pH of the D142E enzyme was practically unchanged from that of the wild-type enzyme when tested using the polysaccharide as the substrate. Presumably, there are subtle differences in the binding of the large and small substrates. Nonetheless, these data suggest that the D142E mutation did not perturb significantly the hydrogen bonding and/or change the interactions between D142 and D144, whereas D142N exhibited a more pronounced effect, resulting in the shift in the optimal pH of the enzyme. Because D142 is rather far from E146 in this triad, mutation of D142 may not have affected the orientation of E146. It is likely that the major effect caused by this mutation was to change the ionization state of E146 indirectly through D144, which resulted in the shift in pH optimum to the acidic range in the D142N mutant and a narrowing of the active pH range of the D142E mutant.

Chitinase B from *S. marcescens* has a homologous catalytic triad of acidic residues, D140, D142 and E144 (Kolstad et al., 2002). The crystal structure of the D140N mutant is essentially identical to that of the wild-type enzyme with the exception of a rotation of D142 in the catalytic center. This mutation disrupts the hydrogen bonding between D140 and D142, which probably perturbs the protonation of E144 and also the ability to bind substrate in a productive manner. It is likely that a comparable situation exists in the D142N mutant of *M. sexta* chitinase, which also results in a localized effect on hydrogen bonding and the rotation of side chain of D144.

4.6. Mutations affect allosamidin inhibition

The IC_{50} of allosamidin for the D144N mutant increased by about 800-fold when compared to the wild-type enzyme. This result demonstrated that the binding of allosamidin was greatly affected by this mutation. The structural similarity between allosamidin and the oxazoline intermediate suggests that the binding ability of the transition state of the substrate to the enzyme also would be impaired by the D144N mutation. This hypothesis also is consistent with the observation that the activity of the D144N mutant was significantly lower than the wild-type enzyme. The mutation D144E also impaired the binding ability of chitinase with allosamidin and, by inference, oxazoline, because the IC_{50} of this mutant form for allosamidin had increased by about 400-fold. The D142E and D142N mutations did not result in dramatic changes in IC_{50} values for allosamidin, consistent with the relatively small effects of these mutations on enzyme activity.

4.7. Mutations affect kinetic parameters

The k_{cat} value of the D144E mutant for the polysaccharide substrate was reduced to about one-third that of the wild-type enzyme, whereas the D144N mutant had only one-eighth of the activity. The other two mutants, D142E and D142N, had activities similar to the wild-type enzyme. Because of compensation due to lower K_m values, the k_{cat}/K_m values for all of the mutant forms remained approximately the same as those of the wild-type enzyme, even though k_{cat} values were quite different. The same result was observed using the oligosaccharide as substrate. Because both substrates are bound by several regions of the enzyme, the binding forces apparently are strong enough to prevent the substrate from diffusing away once the binding occurs. Then cleavage of the glycosidic bond in the polysaccharide/oligosaccharide leads to release of the products. If the catalytic efficiency were reduced as a result of a mutation, a substantial amount of substrate (or transition state) might be trapped by the enzyme, which could greatly reduce the K_m value. A similar kind of situation was observed with mutations of xylanases, where accumulation of the glycosyl-enzyme intermediate occurred with several mutants, which greatly reduced the K_m values.

4.8. Influence of mutations on the pH versus activity profile of M. sexta chitinase

An interesting feature of the pH-activity profiles of *M. sexta* and *B. mori* chitinases is that they are not bell-shaped. They are broad and their shape appears to be determined by multiple ionizable groups. Mutations involving several acidic groups and the tryptophan found in conserved region II resulted in a reduction in specific activity and a shift of the pH optimum to the acidic side, as well as a narrowing of the pH range over which the enzyme is active. *M. sexta* chitinase has a pH optimum of approximately 8.5 when hydrolyzing the polymeric substrate. It exhibits moderate activity even above pH 10. A group with a pK_a estimated to be about pH 9 apparently is influencing the activity-pH profile on the alkaline side. E146 is very likely that residue with an abnormally high pK_a. The role of several acidic residues and W145 in maintaining this alkaline pH optimum was revealed by our mutation studies (this work and Huang et al., 2000). All of our D144 and W145 mutations resulted in a shift in the pH optimum toward the acidic range. The high pK_a value of E146 can be attributed in part to electrostatic repulsion from the two neighboring acidic residues, D144 and D142. Thus, E146 remains protonated under most conditions and essentially resembles a neutral glutamine residue at physiological pH. During catalysis, however, it serves as the proton donor that helps to electrophilically activate the C1 carbon of the scissile bond for nucleophilic attack by the acetamido oxygen atom of the substrate.

The high activity of *M. sexta* chitinase in the alkaline range is critical for the digestion of chitin in vivo, because the hornworm has an alkaline pH in its midgut (pH>10). With such a broad pH range, the insect enzyme is functional not only in the midgut at alkaline pH but also in molting fluid, which has a pH of approximately 7. Although most of the plant, bacterial, and animal chitinases have lower pH optima of approximately 6 (Koga et al., 1997), this kind of alkaline pH activity is not unique. A chitinase from *B. mori,* an insect with an alkaline midgut like *M. sexta* (Koga et al., 1983, 1997), and a thermostable chitinase from an alkalophilic *Bacillus* sp. (Bhushan, 2000) also have alkaline pH optima. A similar shift in the pH optimum from alkaline to acidic pH occurred after mutation of the homologous glutamate residue in *C. fimi* β–glucanase (MacLeod et al., 1994, 1996). All of these data lend strong support to the notion that the pH vs activity profile of chitinolytic enzymes like that of *M. sexta* is a manifestation of a complex function of several interacting residues, some of which have abnormally high pK_a values.

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